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14. ABSTRACT

Identification of novel drivers of cancer is necessary to understand the mechanisms of transformation as well as reveal more efficient targets for cancer therapy. Various subtypes of human cancer show activation of the RAS-MAPK pathway however, activating mutations in the pathway are frequent only in a few subtypes. For instance, although human breast tumors show activated RAS-MAPK signaling, activating mutation of RAS or members of the MAPK pathway is rare, indicating that an alternate mechanism is in play to activate MAPK signaling in this subtype. We conducted a kinase ORF gain-of-function screen to identify kinases that can transform human mammary epithelial cells in a MAPK-dependent manner. We identified three kinases, PAK1, PTK6 and CAMK4, which promoted robust anchorageindependent
growth of HMLE cells. We further showed that PAK1 and PTK6 are amplified in human breast cancer and can activate the RAS-MAPK pathway for transformation. Moreover, PTK6 behaved in a cooperative manner to enhance transformation while PAK1 was shown to be a driver of transformation in mammary cells. Hence, we conclude that amplification of kinases such as PAK1 and PTK6 are alternative mechanisms by which the RAS-MAPK pathway can be activated in breast cancer.

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INTRODUCTION

RAS-MAPK pathway is commonly activated in human cancers. Activating mutations of the pathway are frequently identified in several types of cancer, but the frequency of such in human breast cancer is low. Hence, in this cancer type activation of the RAS-MAPK pathway occurs through a different mechanism. In this project, we investigated the possibility of MAPK activation through overexpression and amplification of kinases. We conducted a kinome screen for anchorage-independent growth of derivatives of human mammary epithelial cells primed for the activation of the MAPK pathway for transformation.

We have previously reported the screen conducted in HMLEA cells in detail. The screen identified three top hits, PAK1, PTK6 and CAMK4 as kinases that promote anchorage-independent growth of HMLEA cells. We have also previously reported the characterization of PTK6 as a collaborative oncogene that activates the MAPK pathway. In this report, we present the data to support PAK1 as a breast cancer oncogene and elucidate its mechanism of action for transformation of mammary cells.

We point out that the Statement of Work was modified for the last progress report and here we present the data for the revised tasks 1, 2 and 3 within the revised statement of work.

BODY

The modified specific tasks/aims from the original Statement of Work for the project are as follows:

Revised Task/Specific Aim 1: Screen for breast cancer oncogenes: *In vitro* and *in vivo* screens

Revised Task/Specific Aim 2: Relevance of kinase hits in human breast cancer: Validation and determination of amplification status of two kinase hits: PAK1 and PTK6

Revised Task/Specific Aim 3: Determine mechanism of oncogenic function for two kinase hits: PAK1 and PTK6

Revised Task/Specific Aim 1 - Completed

Aim 1A: Conduct a pooled kinase ORF screen to assay anchorage-independent growth of HMLEA cells.

Previously reported – We screened the pBabe-Puro-Myr-Flag kinase ORF library containing 597 kinase and kinase-related ORFs in HMLEA cells for anchorage-independent colony formation. Five pools out of 22 showed significant colony formation. These five pools were deconvoluted to individual kinase levels to identify three top kinase hits that promoted robust anchorage-independent colony formation of HMLEA cells. The three kinases are: PTK6, PAK1 and CAMK4.

Aim 1B: Conduct a pooled kinase ORF screen to assay tumor formation in immuno-compromised mice.

Previously reported - We screened the pooled pBabe-Puro-Flag and pBabe-Puro-Myr-Flag kinase ORF libraries in HMLEA cells *in vivo* for subcutaneous tumor formation. We created pools of about 50 kinases per pool and injected HMLEA cells expressing these pools subcutaneously in immuno-compromised mice. The kinase pools did not form any tumors and the *in vivo* screen was not repeated. We believe that the complexity of the pools were too high for a tumor formation screen. In the future a lower dilution of the kinases such that there are larger number of pools in the screen may prove fruitful for an *in vivo* tumor formation screen.

Revised Task/Specific Aim 2

Aim 2A: PAK1 Validation – Completed

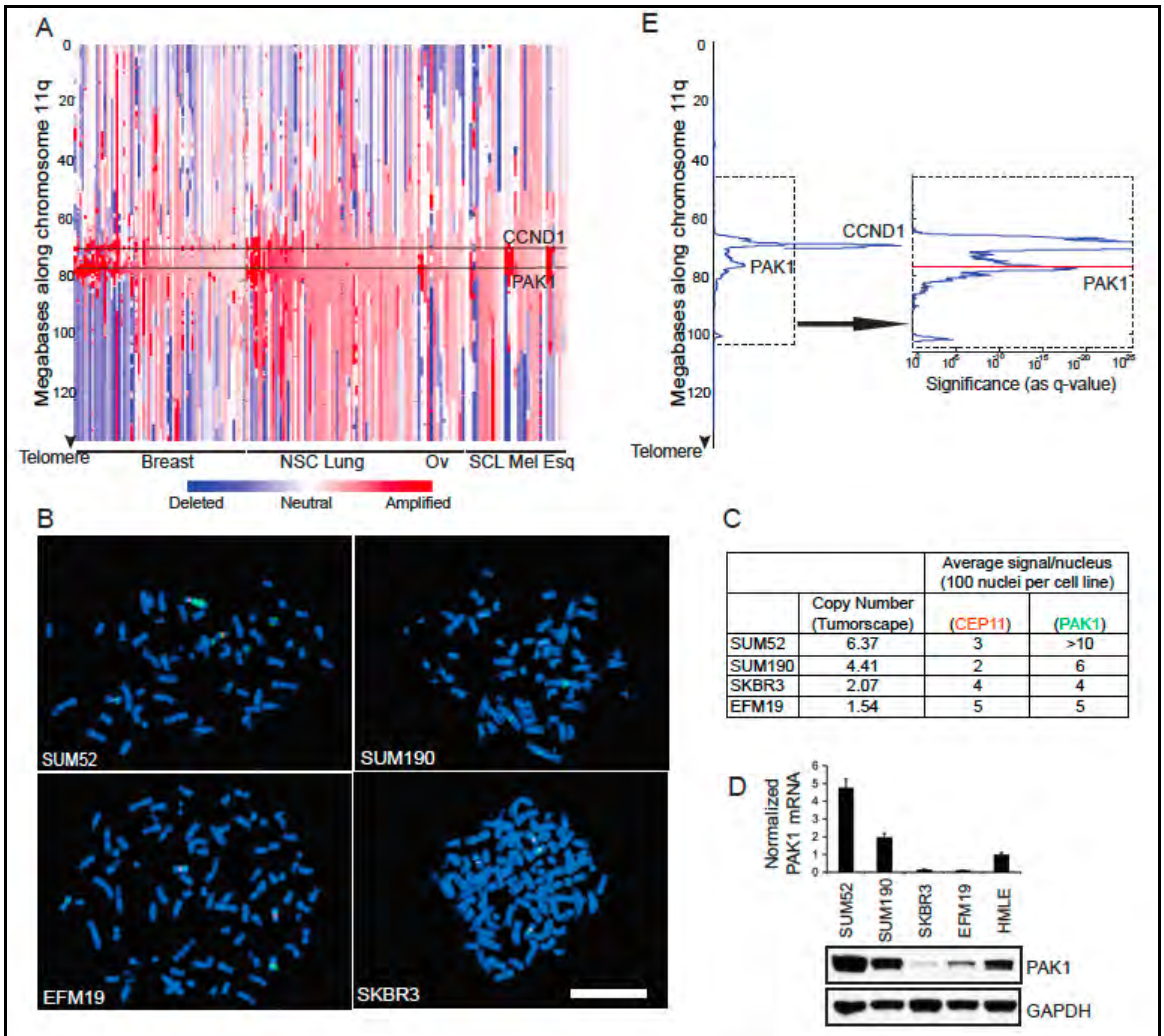
Aim 2A.1: Establish the necessity of myristoylation and kinase activity of PAK1 for anchorage-independent growth of HMLEA cells.

Previously reported – We assessed the effect of myristoylation and kinase activity of PAK1 in transformation of HMLEA cells. Myr-Flag-PAK1 and Flag-PAK1 constructs

were introduced to HMLEA cells to discover no significant difference in soft agar colony formation, indicating that myristoylation of PAK1 is unnecessary for PAK1-dependent anchorage-independent growth. On the other hand, HMLEA cells expressing kinase dead PAK1 (K299R) did not show robust colony formation, in contrast to HMLEA cells expressing wild-type *PAK1*. Thus, the next step in characterizing the role of PAK1 will be to study its activity in various relevant pathways and their contribution in PAK1-dependent transformation. Similar studies will be carried out to understand the mechanism of PTK6 and CAMK4 in transformation of HMLEA cells.

Aim 2A.2: Determine amplification status of *PAK1* in human breast cancer

We used a publicly available dataset called Tumorscape (www.tumorscape.com), which is a study of 3131 human tumor samples and cancer cell lines including 243 breast samples. Tumorscape showed that *PAK1* is located in an amplicon distinct from a neighboring amplicon containing *CCND1* on chromosome 11q (Figure 1A). Furthermore, *PAK1* amplification was most common in breast but was also present in several other types of cancers (Figure 1A). In order to verify amplification of *PAK1*, we used Tumorscape information and conducted FISH analysis in two breast cancer cell lines (SUM52 and SUM190) that exhibited *PAK1* amplification and two other breast cancer cell lines (SKBR3, EFM19) that did not. We found increased signal of PAK1 in SUM52 and SUM190 lines compared to the chromosome 11 reference probe, but not in the other two lines (Figure 1B). Further we show that the copy number of *PAK1* of the cell lines corresponded to the *PAK1* probe signal from the FISH analysis (Figure 1C). The expression level of PAK1 in the four cell lines also corresponded to their copy number status (Figure 1D). Finally, Genomic Identification of Significant Targets in Cancer (GISTIC) analysis of Tumorscape samples indicated that *PAK1* lies on a significant amplification peak that is present in 33% of all samples in Tumorscape that is distinct from *CCND1* suggesting that *PAK1* may be the target of the second peak on chromosome 11q (Figure 1E).



	Copy Number (Tumorscape)	Average signal/nucleus (100 nuclei per cell line) (CEP11)	(PAK1)
SUM52	6.37	3	>10
SUM190	4.41	2	6
SKBR3	2.07	4	4
EFM19	1.54	5	5

Figure 1: *PAK1* is amplified in human cancers. A. Copy number profile along chromosome 11q of human tumor samples and cancer cell lines that exhibit highest level of *PAK1* amplification divided according to cancer type: breast, non-small cell (NSC) lung, ovarian (Ov), small cell lung (SCL), melanoma (Mel) and esophageal squamous (Esq). *PAK1* and *CCND1* loci are marked. B. FISH of breast cancer cell lines with (SUM52, SUM190) and without (EFM19, SKBR3) *PAK1* amplification. Orange fluorescence indicates chromosome 11 centromere reference probe. Green fluorescence indicates PAK1 probe. White bar = 100 μ. C. Tumorscape copy number data is tabulated along with corresponding average number of PAK1 and reference signals from 100 nuclei per cell line. D. mRNA and protein levels of PAK1 in the four breast cancer cell lines and HMLE cells. E. GISTIC analysis of 3131 human cancers along chromosome 11q showing two distinct amplification peaks containing *PAK1* or *CCND1*. The arrow indicates a higher magnification representation of the dotted area to show *PAK1* locus (Red).

Aim 2A.3: Establish necessity of *PAK1* in breast cancer cell lines with *PAK1* amplification

The next step was to determine the essentiality of *PAK1* amplification and expression. We suppressed *PAK1* in a cell line with *PAK1* amplification (SUM52) or overexpressed *PAK1* in an immortalized mammary epithelial cell line (HMLE) to determine its role in proliferation. As shown in Figure 2A, alteration of PAK1 levels in either case did not affect the population doubling rate indicating that PAK1 does not regulate proliferation of these cells lines.

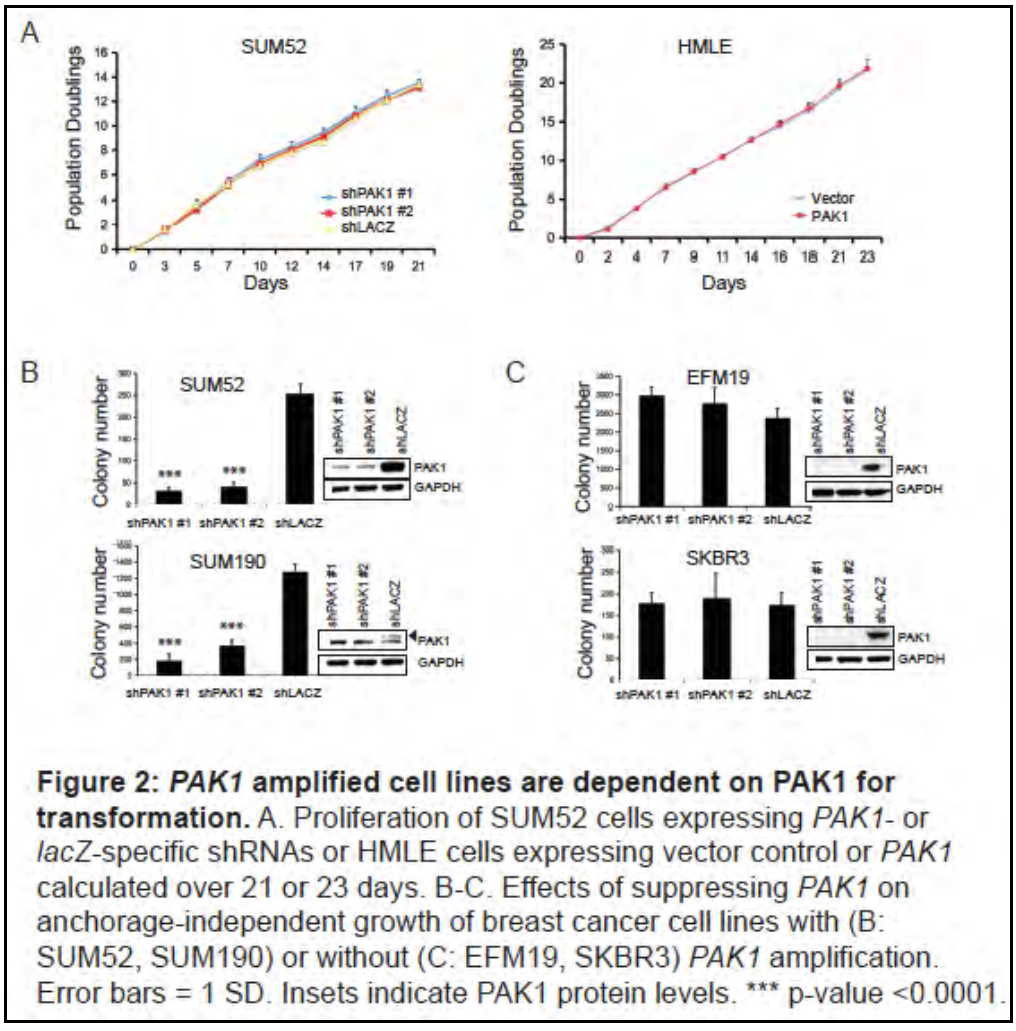


Figure 2: *PAK1* amplified cell lines are dependent on PAK1 for transformation. A. Proliferation of SUM52 cells expressing *PAK1*- or *lacZ*-specific shRNAs or HMLE cells expressing vector control or *PAK1* calculated over 21 or 23 days. B-C. Effects of suppressing *PAK1* on anchorage-independent growth of breast cancer cell lines with (B: SUM52, SUM190) or without (C: EFM19, SKBR3) *PAK1* amplification. Error bars = 1 SD. Insets indicate PAK1 protein levels. *** p-value <0.0001.

We also suppressed *PAK1* in cell lines (SUM52, SUM190) with or (EFM19, SKBR3) without amplification and assessed their ability to grow in an anchorage-

independent manner. Remarkably, we observed that cell lines exhibiting *PAK1* amplification and overexpression were highly dependent on PAK1 for colony formation while cell lines without *PAK1* amplification or overexpression did not show PAK1-dependent colony forming ability (Figure 2B, 2C). Hence, we conclude that PAK1 is essential for transformation of cells exhibiting *PAK1* amplification and overexpression.

Aim 2B: PTK6 Validation - Completed

Aim 2B.1: Establish role of PTK6 in immortalized mammary epithelial cells.

Previously reported - In order to validate the role of PTK6 in transformation of human mammary epithelial cells, we expressed the vector control Myr-Flag-PTK6 or Flag-PTK6 in HMLE cells and assayed for anchorage-independent growth ability of the cells. We observed that unlike in HMLEA cells, PTK6 by itself is not able to significantly increase colony forming ability of HMLE cells. Moreover, the myristoylation of PTK6 did not alter its abilities in this assay.

Aim 2B.2: Determine co-operativity of PTK6 with other signaling pathways.

Previously reported - We investigated the role of PTK6 in anchorage-independent growth of HMLEA as well as HMLEM (HMLE-MEK^{DD}). We expressed Myr-Flag-PTK6 or Flag-PTK6 in HMLEA and HMLEM cells and compared their abilities to form colonies. PTK6 enhanced anchorage-independent colony formation of both HMLEA and HMLEM cells. These results support the idea of PTK6 being a cooperative oncogene that increases the malignancy of transformation. Furthermore, PTK6 expression cooperated with activation of two distinct RAS effector pathways, PI3K and MAPK.

Aim 2B.3: Determine necessity of myristoylation for PTK6-driven anchorage-independent growth.

Previously reported - Furthermore, we observed that myristoylation of PTK6 may contribute to its transforming function. In HMLEA cells myristoylated PTK6 formed more colonies than non-myristoylated PTK6, however, no significant difference was seen between the two in HMLEM cells. Although PTK6 is not a receptor tyrosine kinase and does not have a myristoylation/palmitoylation tag, it has been known to interact with

other kinases at the membrane. Hence, we believe that myristoylation of PTK6 may allow interaction of PTK6 with its activators and effectors at the membrane.

Aim 2B.4: Determine amplification status of *PTK6* in human breast cancer.

Previously reported - Tumorscape copy number analysis along chromosome 20q showed that *PTK6* is amplified in 54% of the breast samples in Tumorscape. GISTIC algorithm showed that the amplification significant (q-value) of *PTK6* in breast samples was 7.77×10^{-8}. We also observed that PTK6 is located in a broad amplicon. Taken together, analysis of Tumorscape shows that low-level amplification of PTK6 is frequent in human breast cancer, further suggesting that PTK6 gain-of-function by amplification may aid in the transformation of other driving oncogenic alterations.

Revised Task/Specific Aim 3 – Completed

Aim 3A: Determine the mechanism of PAK1-driven anchorage-independent colony formation – Completed

With strong evidence of PAK1's role in transformation, we aimed to determine its mechanism of action. Firstly, we investigated the possible role of PAK1 in RAS-MAPK activation. We had previously reported that *PAK1* overexpression increases phosphorylation of RAF1 and MEK1, known targets of PAK1, as well as members of the MAPK pathway. Hence, we checked the level of phospho-ERK1/2, effectors of the MAPK pathway, and determined that PAK1 overexpression can enhance phospho-ERK1/2, and subsequently, MAPK activity (Figure 3A). In addition, suppression of *RAF1* abrogated PAK1-mediated colony formation in HMLE-PAK1 and SUM52 cell lines (Figure 3B). Using a MEK inhibitor, U0126, we observed that cell lines that exhibit PAK1-driven colony formation are highly sensitive to MEK inhibition compared to cell lines that are independent of PAK1 for anchorage-independent growth (Figure 3C). Finally we suppressed *PAK1* in HMLE-RAS^{V12} (HMLER) cells and observed partial inhibition of colony formation (Figure 3D). Hence, our data indicate that PAK1 activates the RAS-MAPK pathway, which is required to mediate PAK1-driven transformation.

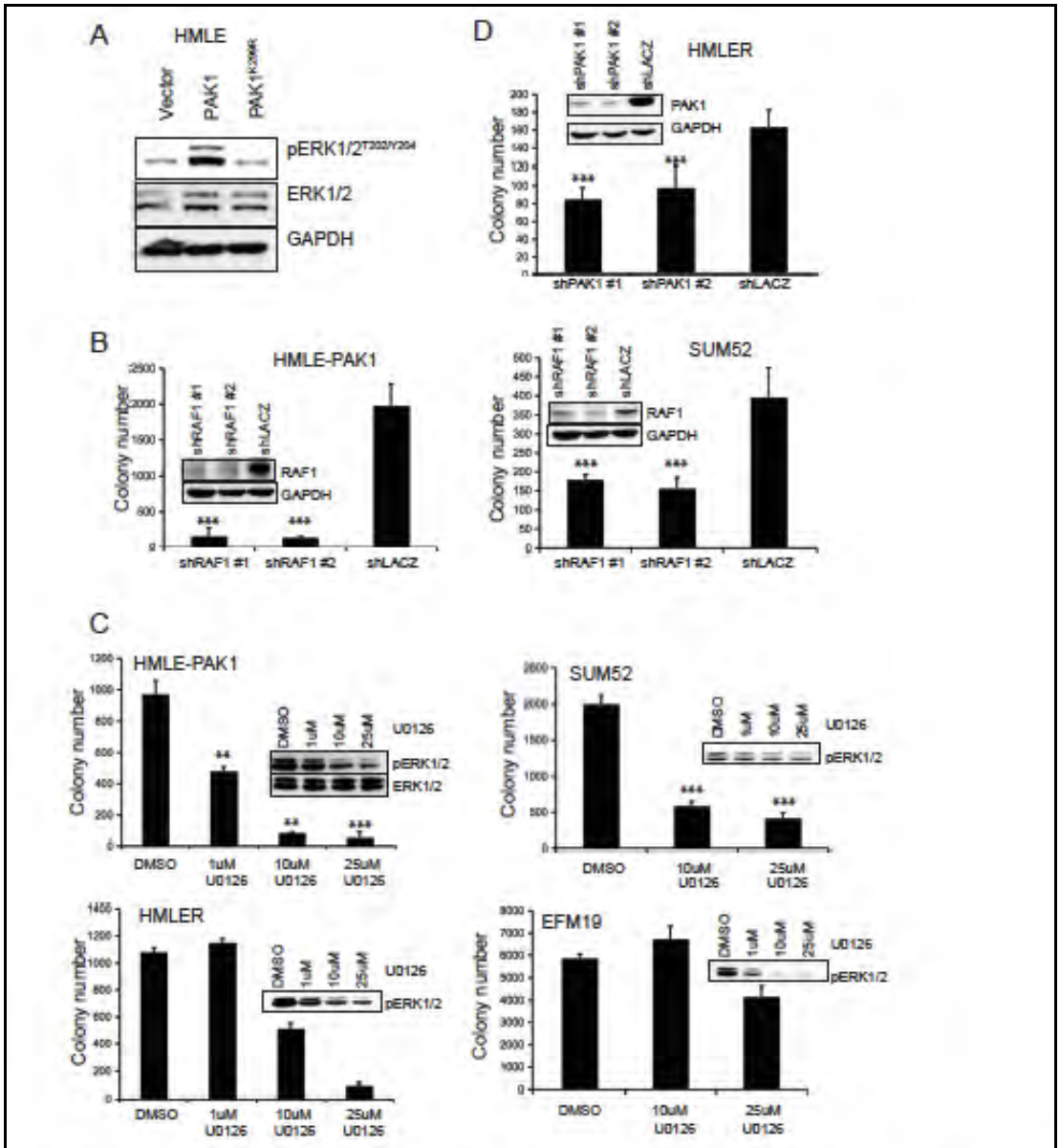


Figure 3: MAPK activation by PAK1 expression is required for anchorage-independent growth. A. Phosphorylation status of the MAPK pathway downstream effectors ERK1/2$^{T202/Y204}$ in HMLE expressing a control vector, PAK1 or PAK1^{K299R}. Total ERK1/2 levels are also shown. B. Anchorage-independent colony formation by HMLE-PAK1 and SUM52 expressing *RAF1*- or *lacZ*-specific shRNAs. Corresponding RAF1 protein levels are shown. C. Anchorage-independent growth of HMLE-PAK1, HMLER, SUM52 and EFM19 treated with U0126 at the indicated doses. Representative phospho-ERK1/2$^{T202/Y204}$ levels are shown. D. Anchorage-independent colony formation by HMLER cells expressing *PAK1*- or *lacZ*-specific shRNAs. PAK1 protein levels are shown. Error bars = 1 SD. ***p-value <0.0001. **p-value <0.001.

We had also previously reported that PAK1 overexpression increases phosphorylation of another target, Merlin, a tumor suppressor. Such phosphorylation of Merlin is known to inhibit its tumor suppressive function. Hence, we investigated the role of Merlin inhibition in PAK1-driven transformation.

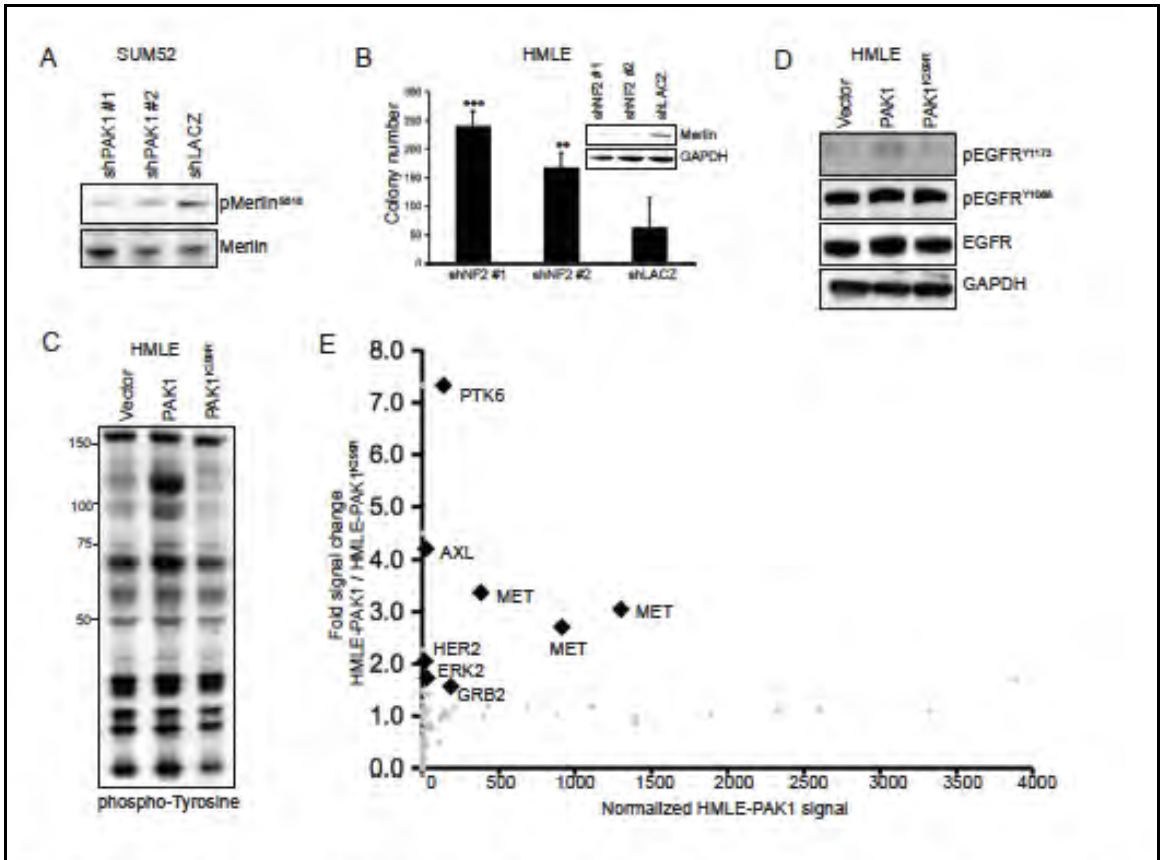


Figure 4: PAK1 expression inhibits Merlin function, subsequently upregulating phospho-tyrosine levels. A. Phosphorylation status of MerlinS518 in SUM52 cells expressing *PAK1*- or *lacZ*-specific shRNAs. B. Anchorage-independent colonies formed by HMLE expressing *NF2*- or *lacZ*-specific shRNAs. Merlin protein levels are shown. ***p-value = 0.0002, **p-value = 0.0059. C-D. Immunoblots showing overall phospho-tyrosine levels (C) or phospho-tyrosine levels of EGFR at Y1173 and Y1068 (D) in HMLE cells expressing a control vector, PAK1 or PAK1 ^{K299R}. E. Luminex assay showing normalized phospho-tyrosine signals of TKs and associated proteins in HMLE-PAK1 (x-axis) and the fold phospho-tyrosine signal increase in HMLE-PAK1 when compared to HMLE-PAK1^{K299R} (y-axis). Each diamond (gray and black) represents individually distinct antibodies. Analytes considered positive (>10) and showing significant (1.5) fold increase in signal are shown as black diamonds. All labeled analytes (black diamonds) scored in lysates harvested in normal growth conditions.

Firstly, we observed that suppression of PAK1 in SUM52 decreased the phosphorylation of Merlin at S518 (Figure 4A) indicating that PAK1 levels may directly

result in inhibition of Merlin function. Thus, when we suppressed Merlin in HMLE cells we observed that it promoted transformation of HMLE cells, suggesting a tumor suppressive role of Merlin in breast cancer (Figure 4B).

A study from the McClatchey group had previously reported that Merlin could regulate overall phospho-tyrosine activity including EGFR and its effectors (Curto et al., 2007). Using a general phospho-tyrosine antibody (4G10) we determined that overexpression of *PAK1* indeed upregulates phospho-tyrosine levels (Figure 4C). However, when we checked phospho-EGFR levels, we did not observe significant changes when *PAK1* was overexpressed (Figure 4D). Hence we conducted a bead-based phospho-tyrosine profile as described in Du et. al., 2009 to determine other possible targets of Merlin inhibition. We observed six kinases whose phosphorylation levels were enhanced due to the expression of wild-type *PAK1* in HMLE cells normalized to kinase-dead *PAK1*. Remarkably, three distinct antibodies identified MET as highly phosphorylated specifically in HMLE-PAK1 cells strongly suggesting that MET could be an alternative target of Merlin (Figure 4E).

We further investigated the role of MET and Merlin in PAK1-driven transformation. We first validated that MET signaling was activated in PAK1 overexpressing HMLE cells. We observed an increase in phosphorylation of MET at two independent sites as well as an increase in phosphorylation of its adaptor protein GAB1 and an effector, STAT3 (Figure 5A). Subsequent expression of Merlin in HMLE-PAK1 cells resulted in a suppression of PAK1 phosphorylation as well as complete abrogation of MET phosphorylation, suggesting that Merlin may indeed inhibit MET signaling (Figure 5B).

After establishing the link between PAK1 and Merlin as well as Merlin and MET, we then investigated the role of MET in PAK1-driven transformation. We thus used a MET inhibitor PHA-665752 to inhibit MET signaling in HMLE-PAK1 as well as SUM52 cell lines and observed that it affected colony formation of these PAK1-dependent cell lines (Figure 5C). We further suppressed MET or GAB1 and noted similar results that led us to conclude that MET signaling through GAB1 is also necessary to complete PAK1-driven transformation (Figure 5D).

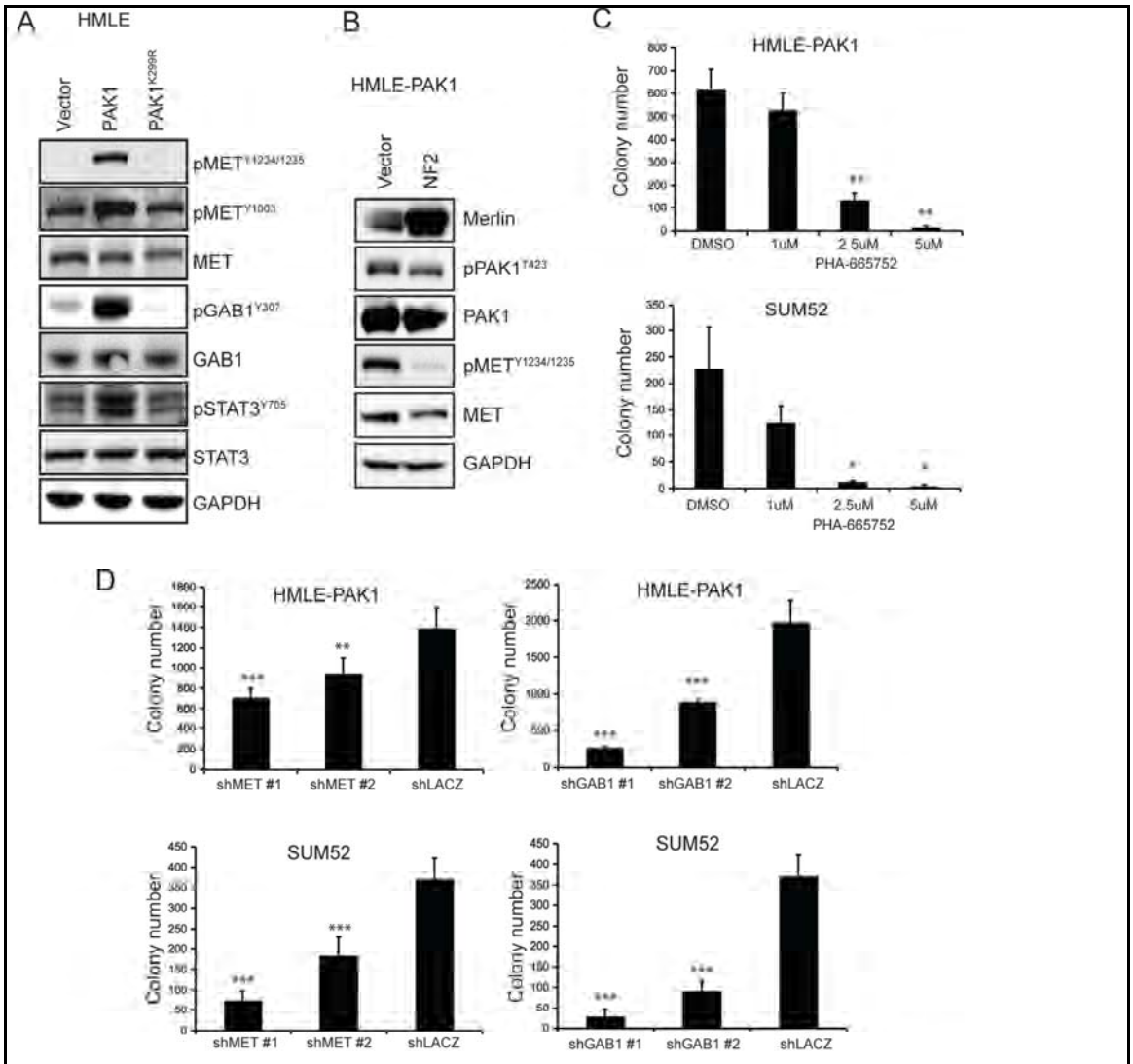


Figure 5: MET signaling activation due to PAK1 expression promotes anchorage-independent growth. A. Phospho-tyrosine levels of MET at Y1234/1235 and Y1003, its adaptor protein GAB1^{Y307} and downstream effector STAT3^{Y705} in HMLE expressing a control vector, PAK1 or PAK1^{K299R}. Corresponding total protein levels are shown. B. Immunoblots of HMLE-PAK1 cells expressing vector control or ^{NF2} showing levels of Merlin, phosphorylated MET at Y1234/1235, phosphorylated PAK1 at T423. Total protein levels are shown. C. Anchorage-independent growth of HMLE-PAK1 and SUM52 after treatment with PHA-665752, a MET inhibitor, at indicated doses. D. Anchorage-independent colonies formed by HMLE-PAK1 or SUM52 expressing *MET-*, *GAB1-* or *lacZ*-specific shRNAs. ***p-value <0.0001, **p-value <0.002, *p-value <0.01.

Aim 3B: Determine the mechanism of PTK6-driven anchorage-independent survival. - Completed

Previously reported - Our collaborators determined that PTK6 is also necessary for anchorage-independent survival of MCF10A cells in an IGF1-dependent manner. MCF10A cells expressing IGF1R can be cultured in suspension in the presence of IGF1. When they suppressed PTK6 with specific siRNAs and shRNAs, they determined that MCF10A-IGF1R cells lose their ability to grow in an anchorage-independent manner. They further showed that PTK6 regulated phosphorylation of IGF1R as well as RAS effectors PI3K and MAPK. Their results indicated that PTK6 regulation of IGF1R and its downstream signaling through RAS effectors may trigger proliferation and survival signals that allow survival and growth of MCF10As in an anchorage-independent manner.

KEY RESEARCH ACCOMPLISHMENTS

- Successfully completed *in vitro* ORF screen for kinases that transform mammary epithelial cells
- Conducted an *in vivo* screen for kinases that promote tumor formation in immune-compromised mice
- Identified kinases that promote robust transformation of human mammary epithelial cells
- Investigated the structural genomics of human tumor samples and cancer cell lines to determine the copy number status of two kinase hits: PAK1 and PTK6
- Initiated a collaboration that led to characterization of the role of PTK6 in breast tumorigenesis
- Established PTK6 as a collaborating oncogene in breast cancer
- Established PAK1 as a breast cancer oncogene that regulates MAPK and Merlin/MET signaling for transformation
- Determined that PAK1 kinase activity requirement for transformation makes it an attractive target for drug inhibition

REPORTABLE OUTCOMES

Dissertation:

Shrestha Y. Deciphering mechanisms of cell transformation through functional genomics. Chapters 2, 3 and 4. April 2011.

Poster:

Shrestha Y, Schafer EJ, Boehm JS, Thomas SR, Weir B, Beroukhim R, et al., Human kinase screen to identify novel breast cancer oncogenes. Era of Hope 2011.

CONCLUSIONS

We have successfully completed an *in vitro* screen to identify novel transforming kinases in human breast cancer. The screen revealed three kinases PAK1, PTK6 and CAMK4 to enhance anchorage-independent growth of HMLEA cells. We validated the role of two candidates PTK6 and PAK1 in transformation. We also characterized the mechanism of action of the two candidates.

During this project, we showed that *PTK6* is a cooperative oncogene that significantly enhances anchorage-independent colony formation by activated RAS effector pathways, PI3K and MAPK. PTK6 by itself cannot transform immortalized human mammary epithelial cells. We also showed that the genomic status of *PTK6* point towards an oncogenic role in human breast cancer.

We have similarly characterized PAK1. Tumorscape analysis showed that PAK1 is significantly amplified in numerous types of human cancers but most significantly in breast tumors. Our subsequent studies provide strong support that *PAK1* is the target in the second amplicon on chromosome 11q. We have shown that PAK1 is essential in samples with amplification for transformation. We further showed that both *PAK1* and *PTK6* amplifications are alternative mechanism by which RAS-MAPK pathway may be activated in human breast tumors that lack activating mutations in the pathway.

Moreover, we show that PAK1 can behave as a regulator of multiple signaling pathways and the cooperation of which translates into robust transformation driven by PAK1.

Hence, we report the successful completion of the project entitled “Identifying breast cancer oncogenes.”

BIBLIOGRAPHY

Curto M, Cole BK, Lallemand D, Liu CH, McClatchey AI. Contact-dependent inhibition of EGFR inhibition of Mf2/Merlin. J Cell Biol. 2007 Jun 4;177(5):893-903.

Du J, Bernasconi P, Clauser KR, Mani DR, Finn SP, Beroukhim R et. al., Bead-based profiling of tyrosine kinase phosphorylation identifies SRC as a potential target for glioblastoma therapy. Nat Biotechnol. 2009 Jan;27(1):77-83.

APPENDICES

Abstract (Era of Hope 2011)

Although RAS signaling pathway is commonly active in breast cancer, activating mutations of RAS and RAF-MEK-ERK effector pathway are rarely found. In these cases, RAS signaling is activated through growth factor receptor upregulation upstream of RAS or effector pathway activation downstream of RAS. For example, a fraction of breast tumors exhibit amplification of ERBB2, which leads to cooperative activation of RAS effector pathways, PI3K and MAPK. Although activation of the MAPK pathway has been observed, activating mutations in MEK or ERK have not been described, suggesting that MAPK activation in breast cancers occur through alternative mechanisms.

We conducted a screen for kinases that activate the MAPK pathway and cooperate with PI3K pathway activation to promote mammary cell transformation. Immortalized human mammary epithelial cells expressing myristoylated-AKT1 (HMLEA) were infected with pooled human open reading frame (ORF) kinase library and assayed for anchorage-independent growth. Among 597 ORFs, twenty-eight

promoted soft agar colony formation of HMLEA cells. These kinase hits were compared to determine three - PTK6, PAK1 and CAMK4 that showed the most robust colony formation.

We determined that PAK1 and PTK6 are amplified in human cancers. We determined that PTK6 behaves as a cooperating oncogene in mammary cell transformation through activation of RAS signaling. In case of PAK1, we have found that kinase-active PAK1 is sufficient for transformation of HMLE cells. Further, PAK1 is required for RAS-dependent transformation. Currently, we are focused on establishing the mechanism of PAK1-dependent transformation.